



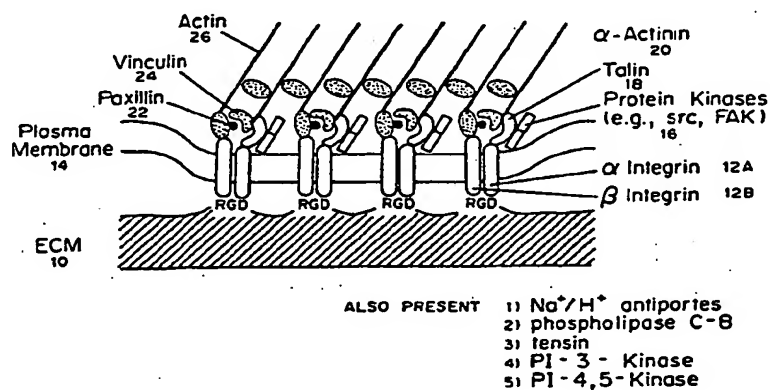
PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification <sup>5</sup> : C07K 15/00, 1/14, 17/14, C12Q 1/68 // C12N 15/12</p>	<p>A1</p>	<p>(11) International Publication Number: WO 94/25487 (43) International Publication Date: 10 November 1994 (10.11.94)</p>
<p>(21) International Application Number: PCT/US94/04481 (22) International Filing Date: 22 April 1994 (22.04.94) (30) Priority Data: 052,895 26 April 1993 (26.04.93) US (71) Applicant: CHILDREN'S MEDICAL CENTER CORPORATION [US/US]; 55 Shattuck Street, Boston, MA 02115 (US). (72) Inventors: INGBER, Donald, E.; 71 Montgomery Street, Boston, MA 02116 (US). PLOPPER, George, E.; 15 Lincoln Street, Cambridge, MA 02141 (US). (74) Agents: PABST, Patrea, L. et al.; Kilpatrick &amp; Cody, Suite 2800, 1100 Peachtree Street, Atlanta, GA 30309-4530 (US).</p>		<p>(81) Designated States: AU, CA, JP, KR, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  Published With international search report. With amended claims.</p>

(54) Title: A METHOD FOR RAPID FORMATION AND ISOLATION OF FOCAL ADHESION COMPLEXES



## (57) Abstract

A method using microbeads coated with specific extracellular matrix ligands, for example, fibronectin or RGD-containing peptide, or any molecule that ligates integrins and thereby mediates attachment and spreading, even through receptors other than integrins, can be applied to the surface of microbeads to induce integrin clustering and isolate functional focal adhesion complexes (FACs) from eukaryotic cells such as mammalian, insect, or plant cells. In the preferred embodiment, magnetic microbeads are utilized. Bead-associated proteins are then isolated and collected for biochemical analysis using a combination of cytoskeletal extraction, sonication, dounce homogenization, and magnetic pelleting of the beads. The FACs are demonstrated to be functionally active and to contain a number of enzymatically and otherwise biologically active proteins. This method for FAC isolation facilitates analysis of molecular interactions and chemical and mechanical signaling mechanisms within functionally intact FACs, independent of global changes in cells shape or cytoskeletal organization. It also serves as a means to isolate and identify proteins not previously known or known to be present in FACs, which can be used to form diagnostics such as peptides, nucleic acid probes or antibodies. The isolated FACs can also be used to screen for molecules which impact on the mechanical and chemical signalling systems within the FACs.

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

## A METHOD FOR RAPID FORMATION AND ISOLATION OF FOCAL ADHESION COMPLEXES

### Background of the Invention

The present invention is generally in the area of protein isolation and is particularly a method for the isolation of biologically active, functional, focal adhesion complexes from eukaryotic cells, the resulting isolated complexes and diagnostics derived therefrom.

The United States government has certain rights in this invention by virtue of National Institutes of Health grants CA-45548 and HL46491 to D.E.Ingber.

Cell adhesion to extracellular matrix (ECM) is required for maintenance of cell growth and function, as reviewed by Ingber, D.E. (1991) Curr. Opin. Cell Biol. 3, 841-848 and Ingber, D.E. and Folkman, J. (1989) Cell 58, 803-805. Cells attach to ECM through binding of cell surface integrin receptors that cluster in localized attachment domains or "focal adhesions". Focal adhesions were originally defined morphologically as regions of the ventral cell membrane that came in closest contact with the underlying ECM and in which actin stress fibers terminated (Cornell, R. (1969) Exp. Cell Res. 58, 289-295; Izzard, C.S. and Lochner, L.R. (1976) J. Cell Sci. 21, 129-159; and Heath, J.P. and Dunn, G.A. (1978) J. Cell Sci. 29, 197-212). However, more recent studies demonstrate that cell adhesion is mediated through formation of a focal adhesion complex (FAC) which contains actin-associated proteins, such as talin, vinculin, paxillin, and  $\alpha$ -actinin (Burridge, et al., (1988) Ann. Rev. Cell Biol. 4, 487-525). FAC proteins interact with the cytoplasmic portion of integrins and thus, physically interconnect ECM with the actin cytoskeleton (CSK). This molecular bridge

-2-

provides a transmembrane path for transfer of CSK tension to the ECM and hence, is critical for cell spreading, migration, and tissue remodeling.

Structural interconnections between integrins and  
5 FAC proteins also mediate mechanosensation, the process by which cells sense and respond to external mechanical signals (Ingber, 1991). In addition, the FAC appears to function as a site for local chemical signaling by integrins (Juliano,  
10 R.L. and Haskill, S. (1993) J. Cell Biol. 120, 577-585; Schwartz, M.A. (1992) Trends Cell Biol. 2, 304-308; and Zachary, I. and Rozengurt, E. (1992) Cell 71, 891-894).

The mechanism of FAC assembly and  
15 organization is poorly understood. Morphological approaches have localized numerous proteins to the FAC, but provide no information on their functional properties or the mechanism of protein-protein interaction. Gel filtration (Burridge, K. and  
20 Mangeat, P. (1984) Nature 308, 744-746; Horwitz, et al., (1986) Nature 320, 531-533), modified gel overlay (Nuckolls, et al., (1990) J. Cell Biol. 110, 1635-1644), recombinant DNA (Bendori, et al., (1989) J. Cell Biol. 108, 2383-2393), and  
25 microinjection (Nuckolls (1990); Pavalko, F.M. and Burridge, K. (1991) J. Cell Biol. 114, 481-491; Schlessinger, J. and Geiger, B. (1983) Cell Motil. 3, 399-403; and Nuckolls, et al., (1992) J. Cell Sci. 102, 753-762) techniques have been used to  
30 identify specific binding interactions between individual FAC proteins, but they fail to address higher order structural interactions within the complex that may be critical for chemical and mechanical signalling. Analysis of FAC assembly,  
35 structure, and function therefore would be greatly facilitated if intact FACs could be isolated in a form amenable to biochemical analysis. An

-3-

isolation method of this type is also critical for development of probes and pharmacological modulators of the chemical and mechanical signaling processes that are critical for control of cell function.

Taken together, the observations suggest that the FAC may represent a cell surface microdomain that functions as a local, solid-state signaling complex. Recently, methods have been described for isolation of ventral cell membranes which retain FAC proteins in relatively high concentrations (Avnur, Z. and Geiger, B. J. Mol. Biol. 153, 361-379 (1981); Brands, R. and Feltkamp, C.A. (1988) Exp. Cell Res. 176, 309-318 (1988); and Gates, et al., Biochem. J. 289, 221-226 (1993). However, these extracts also contain many other CSK and basal cell surface proteins that are not relevant to FAC structure or function. Moreover, these techniques are relatively inefficient in terms of the degree of FAC enrichment which they can accomplish, and no function of any of the resulting compositions has been demonstrated.

Therefore, to understand how ECM exerts its effects, methods that can be used to isolate and analyze how the FAC functions must be developed. It would also be useful to have a method that allowed isolation, identification and characterization of previously unidentified proteins and other types of molecules from the FACs, as well as to serve as a source for nucleic acid probes and antibodies for use as diagnostic indicators.

It is therefore an object of the present invention to provide a method to isolate biologically active, functional focal adhesion complexes from animal and plant cells.

-4-

It is a further object of the present invention to provide a method for the isolation and characterization of previously unknown proteins from functional focal adhesion complexes.

5 It is a still further object of the present invention to provide a method to make, and the compositions thereby derived, probes and antibodies to the previously unknown proteins, associated lipids, gangliosides, complex carbohydrates, and  
10 proteoglycans from functional focal adhesion complexes.

#### Summary of the Invention

A method using microbeads coated with specific extracellular matrix ligands, for example,  
15 fibronectin or RGD-containing peptide, or any molecule that ligates integrins and thereby mediates attachment and spreading, even through receptors other than integrins, can be applied to the surface of microbeads to induce integrin  
20 clustering and isolate functional focal adhesion complexes (FACs) from eukaryotic cells such as mammalian, insect, or plant cells.

Immunofluorescence microscopy reveal that proteins commonly found in FACs, including  $\beta 1$  integrin,  
25 talin, vinculin,  $\alpha$ -actinin, paxillin, and actin, become concentrated along the cell-bead interface within 15 to 30 minutes after bead addition. In the preferred embodiment, magnetic microbeads are utilized. Bead-associated proteins are then  
30 isolated and collected for biochemical analysis using a combination of cytoskeletal extraction, sonication, dounce homogenization, and magnetic pelleting of the beads. The FACs are demonstrated to be functionally active and to contain a number  
35 of enzymatically and otherwise biologically active proteins, including protein tyrosine kinases

-5-

(c-src, FAK kinase), lipid kinases (phosphatidyl inositol kinases), GTP-binding proteins, Na<sup>+</sup>/H<sup>+</sup> antiporter, phospholipase C, and inositol lipids.

This method for FAC isolation facilitates  
5 analysis of molecular interactions and chemical and mechanical signaling mechanisms within functionally intact FACs, independent of global changes in cell shape or cytoskeletal organization. It also serves as a means to isolate and identify proteins not  
10 previously known or known to be present in FACs, which can be used to form diagnostics such as peptides, nucleic acid probes or antibodies. The isolated FACs can also be used to screen for molecules which impact on the mechanical and  
15 chemical signalling systems within the FACs.

#### Brief Description of the Drawings

Figure 1 is a cross-sectional schematic view of a focal adhesion complex.

Figure 2 is a schematic flow chart of the  
20 method of isolation of FACs described herein.

Figure 3 is a bar graph of the time course of FAC assembly for actin,  $\beta$ 1 integrin, talin,  $\alpha$ -actinin, paxillin, and vinculin. The relative rate of recruitment of the different FAC proteins  
25 following cell surface binding to FN-beads was determined by scoring all cell bound beads in at least two different experimental wells and measuring the percentage of beads that exhibited fluorescent staining for the different antigens at  
30 15 and 30 min.

Figure 4 is a bar graph of the FAC protein enrichment during FAC isolation using densitometric quantitation of Western blot data. Densities are expressed as percent of the maximum density  
35 recorded for each antigen: integrin, vinculin, paxillin, talin,  $\alpha$ -actin, and actin. Whole CSK,

-6-

open bars; wet-cleaved basal cell membrane preparation, stippled bars; isolated FAC, black bars.

Figure 5 is a bar graph of the signaling molecules enrichment during FAC isolation using densitometric quantitation of Western blot data. Densities are expressed as percent of the maximum density recorded for each antigen: c-src, Na<sup>+</sup>/H<sup>+</sup>, and PLC. Whole CSK, open bars; wet-cleaved basal cell membrane preparation, stippled bars; isolated FAC, black bars.

Figures 6a and 6b are two dimensional electrophoresis gels. Figure 6a is a gel of the FAC, with proteins not present in, or significantly enriched from, whole CSK circled. Figure 6b is a gel of the whole CSK.

#### Detailed Description of the Invention

Figure 1 is a cross-sectional schematic view of a focal adhesion complex in a mammalian cell. During the method for isolation of FACs described herein, ECM molecules 10 bind to the surface of a microbead on one end and to  $\alpha$  and  $\beta$  integrins 12a, 12b in the plasma membrane 14 on the other. The integrins 12a, 12b are in turn bound to or interreactive with talin 18,  $\alpha$ -actinin 20, paxillin 22, vinculin 24, and actin 26. Protein kinases 16, e.g., src and FAK, also become physically associated with this cytoskeletal framework. Present, but not shown in the figure, are phospholipase C, tensin, PI-3-kinase, PI-4,5-kinase, the Na<sup>+</sup>/H<sup>+</sup> antiporter system, GTP-binding proteins, and inositol lipids, phosphatidylinositol (PI), phosphatidylinositol monophosphate (PIP) and other functionally active molecules. PI, PI kinase, PIP kinase, and diacylglycerol kinase, upstream and downstream members of the inositol



-7-

lipid signaling pathway, are all retained in an active form within isolated FAC. Phospholipase C and protein kinase C are also present in the FAC. At least two of the signaling activities of the isolated FAC, PIP kinase and diacylglycerol kinase, are significantly greater when analyzed *in situ* within the intact FAC than after extraction into solubilization buffer. Enzymes in other pathways have also been demonstrated to be present and active. This provides further support that much of cellular biochemistry and specifically, channeling of lipid signaling (i.e., transfer of molecules to be processed from one functional enzyme to another to another until a final product or end result is produced) occurs in a "solid-state" (on the cytoskeleton with the FAC), rather than in solution.

Although described herein with particular reference to mammalian cells, focal adhesion complexes are present in, and can be isolated from, other types of cells, specifically plant cells and other animal cells. As described by Wagner, et al., Proc. Natl. Acad. Sci. USA 89, 364 (1992) and Wayne, et al., J. Cell. Sci. 101, 611 (1992), plant cells also contain integrins and extracellular matrix molecules that interact in the same ways as those of mammalian origin. For example, vitronectin is present in plant cells and its function is related to development. Disruption of integrins disrupts sensitivity to gravity of the plants. The FACs are isolated from the plant cells after disruption of the plant cell walls using standard techniques such as enzymatic treatment to yield spheroplasts.

Isolation of the FACs using the method described herein results in a functional complex that contains proteins that have not previously

-8-

been isolated, enzyme systems including both enzymes and substrates that are functionally active, cofactor-protein complexes that would be dissociated by standard purification techniques, regulators and regulatory molecules, and binding proteins in association with their substrates. Isolation of the intact complex allows study of these systems in their physiological context and provides a means for looking at the effect of exogenous regulators. It also allows study of molecules whose activity is different within the context of the intact FAC, as compared with the activity of the isolated molecule.

Importantly, isolation of the intact, functional complexes allows studies of the effects of compounds ranging from oligonucleotides and peptides to organic or inorganic molecules on the isolated functional complexes. The effects of the compounds on both chemical signalling and mechanical signalling can be determined. Mechanical signalling involves structural interactions and protein interactions between integrins and cytoskeletal associated proteins, including those forming the microfilaments (actin and myosin), the microtubules (tubulin), and those forming the intermediate filaments (vimentin in endothelial cells, keratin in epithelial cells, desmin in smooth muscle cells, and neural fibrillar protein in neural cells). Mechanical signaling requires that the molecules form a continuous series of molecular linkages that can bear mechanical loads and transmit force from integrins at the cell surface to actin microfilaments within the internal cytoskeleton. Local spatial arrangements may be equally important for enzymes, such as lipid kinases, and cytoskeletal-associated (solid state) lipid substrates, e.g. PI or PIP,

-9-

which are present in the FAC even after extraction with non-ionic detergents. Chemical signalling involves interactions of enzymes with substrate, including reactions involving interactions of multiple enzymes with multiple, interrelated substrates, and pathways relating to cell growth, adhesion, and proliferation.

#### Methods and Materials for Isolation of Functional Focal Adhesion Complexes.

A protocol for the rapid inducement of the formation of chemically and mechanically functional FACs in animal or plant cells, independent of cell spreading and within minutes after integrin ligation, using ECM-coated magnetic microbeads, has been developed, as shown in Figure 2, and expanded. This method is in complete contrast with those previously used since it can be used to study early signaling events, as well as late signaling events. The time course for the late signaling can be monitored by adding beads to non-adherent cells and then collecting beads at different times, and used to identify time or stage-specific signaling molecules. In addition, this method can be used with adherent cells or cells in suspension, independently of cell spreading.

#### Formation of FACs on the surface of microbeads.

Cells are mixed with microbeads coated with FAC-inducing molecules, defined herein as any molecule that ligates integrins and thereby mediates attachment and spreading, even through receptors other than integrins. The mixture is mechanically agitated (using a commercially available orbital shaker, rotator, or Nutator, i.e., rotated around, with or without simultaneous up and down motion) for a period of time. At short times, the efficiency of binding is lower; at longer times, early signaling cannot be detected.

-10-

37°C is the optimum temperature for binding, although studies can be done at any temperature at which the cells will survive. Rotation insures that all cells contact the beads; however, if it is  
5 too fast, it will kill the cells, therefore it is adjusted to minimize membrane breakage and loss of cell viability.

The beads bind to the surface of the cells. Focal adhesion complexes (FACs) containing integrin  
10  $\beta_1$ , talin, vinculin, talin,  $\alpha$ -actinin, and paxillin form within 15 minutes when round cells bind magnetic microbeads coated with integrin ligands, such as fibronectin (FN) or RGD-containing peptide, but not when coated with acetylated-LDL (AcLDL).  
15 AcLDL is a control molecule that is a ligand for a specific transmembrane receptor, the "scavenger receptor", which these cells express on their surface but which does not mediate cell attachment to ECM or ECM-dependent chemical signaling.

20 Isolation of FACs from the surface of microbeads.

Newly formed FACs are isolated and collected for biochemical analysis using a combination of extraction with non-ionic  
25 detergents, sonication, dounce homogenization, and magnetic pelleting, or equivalent procedures for separating the FACs from the remainder of the cell and cytoskeleton. Extraction with a non-ionic detergent is used to remove membranes and soluble  
30 proteins. Although 0.5% Triton X100 is preferred, other non-ionic detergents such as NP40, can be used in buffers which maintain the structural integrity of the actin cytoskeleton after removal of membranes and soluble cytoplasmic components.  
35 Sonication is done until mechanical breakage and disruption of the remaining cytoskeleton bound to the beads is accomplished. The homogenization is used to remove residual large pieces of

-11-

cytoskeletal-nuclear matrix lattice, defined as structures visible extending from the surfaces of the beads under standard phase contrast microscopy, and nuclei. In the preferred embodiment, magnetic pelleting is then used to pellet the beads with the FACS bound to them while the other materials are washed away, although non-magnetic beads can also be used and collected by sieving.

In the most preferred method, cells dispersed with trypsin-EDTA are washed twice in 1% BSA/DMEM, placed in polypropylene tubes (Costar), suspended to a concentration of  $1 \times 10^6$  cells/ml in defined medium containing magnetic RGD beads at a concentration of  $2 \times 10^7$  beads/ml, and rotated for 30 min at 37°C. RGD-coated beads are chosen for large scale isolation of FACS because they exhibit less non-specific clumping during magnetic pelleting. Microbeads and bound cells are collected by placing a magnet at the side of the tubes, suspending in ice cold CSK extraction buffer (CSK-EB: 0.5% Triton X100, 50 mM NaCl, 300 mM sucrose, 3 mM  $MgCl_2$ , 20  $\mu$ g aprotinin/ml, 1  $\mu$ g leupeptin/ml, 1  $\mu$ g pepstatin/ml, 1 mM PMSF, 10 mM PIPES, pH 6.8) lacking the detergent, Triton X100, and transferred to 5 ml polystyrene tubes (VWR). All subsequent procedures are carried out at 4°C using a side pull magnetic separation unit (Advanced Magnetix). The magnetic bead pellet is then transferred into complete CSK-EB plus detergent, sonicated for 10 sec at an output setting of 4, output power 10%, XL2005 cell disrupter, Heat Systems, and homogenized in a 2 ml dounce homogenizer for 20 strokes. The microbeads are magnetically pelleted and washed five times with CSK-EB prior to use.  $1 \times 10^8$  cells yields 1 mg FAC protein.

-12-

Isolated FACS are greatly enriched for all FAC proteins when compared with either the whole cytoskeleton or basal cell membranes whereas actin (a general cytoskeletal marker) is relatively depleted. The FAC is highly enriched in  $\beta_1$  integrin, talin, and vinculin and contains active lipid and protein kinases, thereby allowing detailed molecular analysis of the biochemical properties and functions of intact FACS.

10        FAC-inducing Molecules.

Any molecule that ligates integrins and thereby mediates attachment and spreading, even through receptors other than integrins, can be applied to the surface of microbeads to induce formation of the functionally active FACS. Examples include proteins and peptides characterized by inclusion of attachment sites, such as RGD, REDV, and YIGSR, for example, any other ECM molecules including fibronectin (FN), laminin, vitronectin, fibrinogen, and collagens, and portions and derivatives thereof containing an attachment site, toxins such as the forty amino acid echistatin, and antibodies to integrins. Other molecules that can be used include ligands for cell surface heparan sulfate proteoglycans. Certain cells require the heparin-binding domain of fibronectin in addition to the RGD domain in order to organize focal adhesions and spread. Examples include antibodies to heparan sulfate, heparin-binding peptides such as protamine, synthetic peptides, growth factors such as TGF- $\beta$  and FGF, and some viral proteins. Sugars, including UDP-galactose which binds to cell surface glycosyltransferase, peptides including the sequence YIGSR (bound by laminin receptors), and receptor-specific antibodies can also be used.

-13-

Microbeads.

Superparamagnetic beads are preferred for use in the isolation of FACs since they rapidly demagnetize when the external magnetic field is removed. This greatly facilitates all of the washing steps during purification. However, ferromagnetic microbeads could be used by degaussing the beads to remove their inherent field during the wash steps. Non-magnetic beads can also be used in combination with sieves for collection during washing, although this again is not preferred in terms of ease in handling.

The bead size must be less than the diameter of the cell but large enough to stay on the cell surface during FAC formation and binding, generally in the range of between 0.1 and 50 microns, most preferably approximately five microns.

Molecules are applied to the surface of the microbeads using conventional chemistry for attachment of proteins, such as absorption in a high pH carbonate buffer, crosslinking with glutaraldehyde, carbodiimide, and tosylation. Methods for Characterization of FACs and for Identification and Isolation of Proteins and other Molecules from FACs which have not previously been identified.

The isolated FACs are characterized based on biological activity, composition, and morphometric analysis, as described in more detail in the following examples. FACs are demembrated using detergents during their isolation. However, even after removal of both membranes and soluble cytoplasmic components, FACs retain specific lipids, such as PI and PIP, as well as many functional enzymes. Examples of active enzymes include protein kinases, lipid kinases and phospholipases. In addition, other chemical

-14-

signaling molecules include the cell surface Na/H+ antiporter and various small molecular weight GTP-binding proteins (G proteins) physically associated with the FAC. In other words, the FAC and its  
5 cytoskeletal protein backbone represents a "solid-state" signaling matrix which contains both enzymes and substrates (e.g., PI and PI kinases) in close proximity; a situation which is apparently physiologically very relevant for control of cell  
10 function.

Methods such as two dimensional gel electrophoresis and western blotting demonstrate that the components of the RGD-bead-associated complex are enriched for FAC proteins, including  $\beta 1$   
15 integrin, talin, vinculin, paxillin, and  $\alpha$ -actinin, when compared with extracts of the whole cell cytoskeleton or with a crude preparation of basal cell membranes. Morphometric analysis of the time course of recruitment suggests that the FAC  
20 assembles sequentially from the cell surface inwards: recruitment of  $\beta 1$  integrin > talin >  $\alpha$ -actinin > paxillin >> vinculin, although a crescent of cortical actin staining is seen from the earliest times after bead binding. Similar  
25 actin staining is observed in cells bound to beads coated with the non-adhesion receptor ligand, acetylated low density lipoprotein (Ac-LDL); however, FAC proteins were not recruited to these beads. Other methods include immunofluorescence  
30 staining, enzyme and lipid assays, and screening with DNA probes and antibodies from known and unknown proteins.

In general, the FACs are isolated on magnetic beads as described generally above. A  
35 number of techniques can then be used to identify those components of the FACs that are unknown. In general, these techniques are used to screen and



-15-

isolate these proteins away from components which are also found in cytoskeletal or membrane fractions.

A simple way to screen for novel proteins is to separate and compare proteins from isolated FACs and whole cytoskeleton using two dimensional gel electrophoresis. When the stained gels are compared, proteins that are enriched or specific for focal adhesions can be easily recognized as stained protein spots that appear in the FAC gel but not in a comparable location, based on similar molecular weight, electrophoretic mobility and charge, in the whole cytoskeleton gel. Western blots with antibodies to the known components can also be used to screen for novel proteins by excluding the known components.

#### Methods for Making Diagnostics based on the Newly Identified Proteins.

Once the known and unknown proteins are identified, the unknown, or enriched, proteins can be isolated and identified by protein sequencing. For example, proteins within unstained two dimensional gels can be transferred via immunoblotting to PVDF paper using standard Western blotting techniques, to obtain around 50 picomoles per spot or pool of spots from several gels. The blot is then stained with Ponceau S stain for 90 seconds, washed ten times in an eppendorf tube (1.5 ml) with HPLC grade water, and the spots of interest, determined as described above using parallel stained gels, are cut out using a sterile scalpel. The spots are pooled in sterile plastic vials and allowed to dry at room temperature until "bone dry". Protein microsequencing is then carried out using standard protocols. Once sequence is obtained, it is possible to: determine whether this is a novel protein by comparing the sequence with that of known proteins listed within

-16-

computer data banks; develop specific antibodies against synthetic peptides with this sequence of interest; generate nucleic acid probes which code for this sequence; amplify the encoding sequence  
5 using polymerase chain reaction (PCR) technology or other methods for nucleic acid amplification; pull out larger related DNA sequences and eventually clone the gene encoding the entire protein; and synthesize pertinent anti-sense and sense nucleic  
10 acid probes.

Although described with reference to the isolation of single protein spots of interest, the entire purified FAC can be used to immunize animals for development of specific antibodies, or tested  
15 for enzyme or binding activity, as described below.

**Methods for making Nucleic Acid Probes.**

Once the isolated protein is even partially sequenced, the cDNA can be obtained by PCR amplification and sequenced using the method of  
20 Sanger, et al., "DNA sequencing with chain-terminating inhibitors", Proc. Natl. Acad. Sci. USA, 74:5463-5467 (1977) for comparison with known sequences. If appropriate, the cDNA is radiolabelled with 5'-[<sup>32</sup>P] dCTP to a specific  
25 activity of 1-3 x 10<sup>9</sup> cpm/μg DNA using a random-primer DNA labelling kit following the manufacturer's protocol. These probes are then used to screen either genomic DNA or a specific cell library, such as a lambda gt11 bovine aortic  
30 endothelial cell cDNA library which can be purchased from Clontech, CA.

Briefly, lambda phage is plated on a lawn of Y1090 cells at a density of 5 x 10<sup>4</sup> pfu/150 mm petri dish. Plaque lifts are carried out in  
35 duplicate using nitrocellulose filters as described by Grunstein, W. and D. Hogniss, Proc. Natl. Acad. Sci. USA 74:5463 (1977). Colonies on the filter

-17-

are lysed, and the DNA fixed to the filter by baking. The DNA immobilized on the filters is hybridized to the  $^{32}\text{P}$ -labelled probe and the filter monitored by autoradiography. Colonies whose DNA gives a positive autoradiographic result are recovered from the master plate and, following a second screening, DNA from these positive colonies are isolated using standard protocols, for example, as described by Maniatis, T., E.F. Fritsch and J. Sambrook, Cold Spring Harbor Laboratory, N.Y., 250-270 (1982). This procedure should yield DNA in sufficient quantity and purity for analysis by restriction endonuclease digestion, gel electrophoresis, Southern blotting, and DNA sequencing as well as for subcloning of DNA fragments into plasmid vectors.

Once the plasmid DNA is isolated, standard procedures are used to construct maps of sites cleaved by restriction endonucleases, as described by Maniatis, et al. The complete nucleotide sequence of the longest clone and any others of interest is determined by the dideoxy method, a widely used method for determining DNA nucleotide sequences. This system makes use of the 2', 3'-dideoxy and arabinonucleoside analogues of the normal deoxynucleoside triphosphates, which act as specific, chain-terminating inhibitors of DNA polymerase. This technique has been used successfully in sequencing the DNA of many proteins and is more rapid and accurate than most other methods including the "plus and minus" method of Sanger and Coulson, "DNA sequencing with chain-terminating inhibitors", Proc. Natl. Acad. Sci. USA, 74:5463-5467 (1977) and Sanger and Coulson, "A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase", J. Mol. Biol. 94:441-448 (1975). The dideoxy method can be

-18-

utilized, either after subcloning restriction fragments into an appropriate vector such as the plasmid M13 tg130 or tg131 (Amersham) and transformation into *E. coli* JM101 or by sequencing directly on the plasmid DNA, approaches described by Messing and Vieira, "A new pair of M13 vectors for selecting either DNA strand of double-digest restriction fragments" Gene 19:269-277 (1982) and "The pUC plasmids, an M13mp 7-derived system for insertion mutagenesis and sequencing with synthetic universal primers" Gene 19, 259-268 (1982).

Once isolated and sequenced, the cDNA can be inserted into an appropriate vector such as the *E. coli* expression vector pMG196 which has been successfully used by Docherty et al. in the expression of a human amniotic fluid metalloproteinase inhibitor, "Sequence of human tissue inhibitor of metalloproteinases and its identity to erythroid-potentiating activity" Nature 318:66-69 (1985). *E. coli* is used as the non-mammalian organism of choice for the expression of the proteins in order to take advantage of the extensive recombinant technology that has been developed for this organism. In this plasmid, the trp promoter used to drive transcription of the inserted introduction increases the copy number to 100-200 per chromosome. cDNA can also be inserted into a vector such as the bovine papillomavirus (BPV)-based vector designed for expression in mammalian cells, for example, mammary fibroblast tumor cells (C127 cells). These cells would then be transfected with the plasmid by calcium phosphate coprecipitation and CdCl<sub>2</sub> and ZnCl<sub>2</sub>-resistant foci selected. Secretion by the cells of the selected protein is confirmed after growth in serum free media by Western blot analysis and

-19-

comparison with the isolated protein using antibody to the protein and other standard analytical tests.

#### Methods for making Antibodies.

Methods for making polyclonal and  
5 monoclonal antibodies are now well known to those skilled in the art. In general, antigen, the target of the antibodies to be generated, is injected alone or in combination with an adjuvant such as complete Freund's adjuvant into mice,  
10 usually peritoneally, in intervals of ten days to three weeks, or until sufficient antibody titers are achieved. In the event the protein is not particularly antigenic, it can be chemically coupled to a carrier protein such as bovine serum  
15 albumin (BSA) or tetanus or diphtheria toxin to enhance the immunological reaction against the protein. For example, isolated protein spots on PVDF paper can either be implanted directly (subcutaneously) in the backs of mice in  
20 combination with Freund's adjuvant or beads containing bound FACS or isolated FACS proteins, removed from the bead by elution with RIPA buffer, can be injected into the peritoneal cavity of mice.

The polyclonal antibodies are then  
25 extracted using methods such as ammonium sulfate precipitation and/or ion exchange or affinity chromatography.

Monoclonal antibodies are generated by removal and dissociation of spleen cells, fusion  
30 with an appropriate myeloma cell strain in culture using an agent such as polyethylene glycol, and screening for production of clones producing good titers of antibodies specifically directed against the antigen.

35 For example, monoclonal antibodies can be prepared using the method of Narula, et al., "Identification of a 200-kD, Brefeldin-sensitive

-20-

Protein on Golgi Membranes" J. Cell Biol. 117(1), 27-38 (1992). Briefly, approximately 100 µg FAC proteins would be purified, emulsified in complete Freund's adjuvant and injected intraperitoneally  
5 into Robertsonian 5BnR mice, which will be later boosted twice with the same antigen. Spleen cells from the immunized mice will be fused to FOX NY myelomas using standard procedures and hybridomas will be selected at AAT medium. Culture  
10 supernatants will be screened by immunofluorescence staining on cells bound to RGD-beads.

In the event that a peptide fragment is to be the antigen, it can be synthesized by solid phase methods as described by Rivier, et al.,  
15 Biopolymers 17, 1927 (1978) using an automated Applied Biosystems 430A peptide synthesizer. The synthetic peptide is checked for purity by HPLC on a C8 column and its composition tested by amino acid analysis, as described by Kohn and Langer, J.  
20 Am. Chem. Soc. 109, 817-820 (1987). For the purpose of linking to a carrier protein such as keyhole limpet hemocyanin (KLH), the peptide is synthesized with the cysteine at the carboxy-terminal end. The synthetic peptide is conjugated  
25 to the carrier keyhole limpet hemocyanin (KLH) using maleimido-hexanoyl-N-hydroxy-succinimide ester (MHS) as a heterobifunctional cross-linking reagent, as described by Waszinski, et al., Clin. Phys. and Biochem. (1992). Animals such as eight  
30 pound male New Zealand white rabbits are injected at multiple dorsal intradermal sites with 500 µg each of KLH-peptide conjugate emulsified in complete Freund's adjuvant and are boosted at 6 week intervals with 200 µg of KLH-peptide conjugate  
35 emulsified in incomplete Freund's adjuvant. The rabbits are bled from a central ear vein at various time intervals following initial immunization and

-21-

subsequent booster injection. Sera are aliquoted and stored at -20°C. Positive immunoreaction as determined by ELISA and immunoblot analysis are obtained as described by Wadzinski, et al.

5           Antibodies can be made against proteins isolated from the FACs or against proteins within the context of the FACs. Epitopes can be formed by the mixture of FACs components that will not be present in a simple mixture of the protein  
10 component(s) of the FACs.

          The resulting antibodies can be screened against the bead complex by immunofluorescence staining to identify antibodies that specifically recognize proteins that localize within the FACs in  
15 situ. Alternatively, western blots can be carried out on proteins separated by two dimensional gels to identify probes that recognize specific proteins that are enriched in the FAC fraction relative to the whole cytoskeleton.

20   **Methods for Screening for Compounds having a direct effect on one or more components of, or the function of the whole, FAC.**

          The cytoskeleton contains three principle filament systems: actin-containing microfilaments,  
25 tubulin-containing microtubules, and intermediate filaments which contain vimentin, keratins, desmin, or neurofibrillary proteins, depending on the cell type. The FACs serve as the structure which physically interconnects the intracellular  
30 cytoskeleton with the extracellular matrix and hence transmits mechanical forces across the cell surface. At the same time, the FAC cytoskeletal backbone acts as a orienting scaffold for much of the cells chemical signaling circuits that  
35 interplay both chemically and mechanically, i.e., they are sensitive to both chemical and mechanical changes in the local microenvironment of the FAC.

-22-

Thus, elements that form the structural skeleton that physically interconnects ECM with the cytoskeleton will likely be critical for mechanical signaling (e.g., mechanosensation, proprioception, baroreception, touch, hearing, gravity sensation, etc.) as well as cell shape control. At the same time, chemical signaling molecules that bind and associate on this scaffold will be critical for both control of cell growth and function as well as local modulation of mechanical signaling (i.e., second messenger release will modulate the structural integrity of the backbone itself). For these reasons, identification of proteins that either bind to the FAC or modulate the function of signal molecules within the FAC may be critical for studying and controlling cell function.

Compounds that bind to the FAC can be identified in a number of ways. For example, radiolabelled compounds, e.g., drugs, or biosynthetically labelled cell extracts can be incubated with magnetic beads containing bound FACs for greater than one second at 37°C. The beads and bound proteins are magnetically pelleted and then washed repeatedly in phosphate buffered saline, PBS. The bound radiolabelled protein is removed from the beads using either RIPA buffer, SDS sample buffer, high salt concentration, low pH or any other solution that disrupts protein-protein interactions. Radiolabelled FAC binding proteins are then identified by one or two dimensional gel electrophoresis. If the protein is unknown, e.g., biosynthetic labelled cytoplasmic extracts were used from the start, then the protein spots will be isolated and sequenced as described above. Alternatively, column chromatography can be carried out using the beads and bound FACs as the gel bed and running over large amounts of unlabelled or



-23-

labelled protein extracts and then eluting with solutions of increasing ionic strength.

Compounds which influence the function of the FAC can also be tested using a variety of assays that measure both chemical and mechanical signalling functions of the FAC. Chemical function assays include *in vitro* assays for protein phosphorylation, lipid kinase activity, phospholipase activity and G protein function. For example, a prototype compound which is known to alter cell function can be tested to determine whether its target is located within the FAC. If so, this target can be identified, sequenced, and cloned. At the same time, many other molecules can be screened using the same assay to identify different modulators of the same molecular target as well as modulators of upstream regulators. Once the target signaling protein is characterized, the same approach can be used to identify its downstream target, and so on.

To determine the effect of a compound on cells, one must first treat the cells with the compound and look for an effect immediately after integrin binding. Corresponding studies with the isolated FACs, or FACs bound to the magnetic microbeads, can also be done which provides additional information and a way to extrapolate from *in vitro* (i.e., isolated) studies to *in vivo* studies. For example, the release of initial messengers immediately following binding can be measured.

Examples of compounds that will have an effect on the activity of the FACs enzyme and cofactor systems include peptides derived from the isolated proteins, and oligonucleotides prepared using the isolated cDNA sequences described above. In general, these oligonucleotides will be at least

-24-

14 to 17 nucleotides long, so that they hybridize to the genomic DNA or RNA under physiological conditions as well as under stringent hybridization conditions. These can also be used to generate antisense using standard techniques, which can be introduced into the cells from which the FACs are derived. Peptides of as little as a few amino acids, more preferably from seven to ten amino acids, can be used to inhibit binding of enzymes and cofactors to their natural substrates, using known technology.

The general methods for isolation and characterization of FAC will be more fully understood by reference to the following non-limiting examples.

**Example 1: Isolation of Focal Adhesion Complexes (FAC).**

Magnetic microbeads coated with specific integrin ligands permit rapid induction of FAC formation as well as isolation of newly formed FACs from the remainder of the cell surface and CSK.

The following abbreviations are used: FAC, focal adhesion complex; FN, fibronectin; AcLDL, acetylated-low density lipoprotein; ECM, extracellular matrix; CSK, cytoskeleton; CSK-EB, cytoskeletal extraction buffer; TBS, tris-buffered saline; PIPES, 1,4 piperazinediethanesulfonic acid; DMEM, Dulbecco's modified Eagle's medium; PMSF, phenylmethyl sulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; PBS, phosphate buffered saline; BSA, bovine serum albumin; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

**MATERIALS AND METHODS**

**Experimental System.** Bovine capillary endothelial cells were isolated, cultured and serum-deprived prior to experimental manipulation, as described by Ingber, et al., (1990) J. Cell

-25-

Biol. 100, 1803-1811, the teachings of which are incorporated herein. Quiescent cell monolayers were dissociated by brief exposure to trypsin-EDTA and plated ( $7.5 \times 10^3$  cells/cm<sup>2</sup>) in defined medium, described by Ingber, et al., (1990), on 8-well glass culture slides (Labtek) coated with a low density (25 ng/ml) of fibronectin (Cappel) that promotes cell attachment but not cell spreading, also as described by Ingber, et al., (1990). Briefly, cells isolated from bovine adrenal cortex were grown at 37°C in 10% CO<sub>2</sub>/90% air and passaged on gelatin-coated tissue culture dishes or roller bottles (Costar, Cambridge, MA) in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum, 2 mM glutamine, 100 U/ml penicillin, 100 U/ml streptomycin (Gibco, Grand Island, ME), 20 mM HEPES pH 7.4 (Sigma), and 10 µg/ml endothelial mitogen (Biomedical Technologies, Inc., Stoughton, MA). Two days prior to transferring to defined medium for experiments, cells were incubated in DMEM containing 20 mM HEPES, 1% calf serum, and no endothelial mitogen to deplete exogenous growth factors and induce quiescence.

For experiments, cell monolayers were dissociated by brief exposure to trypsin-EDTA (Gibco), suspended in DMEM containing 1% bovine serum albumin (BSA; Fraction V, Intergen) and 20 mM HEPES, counted using a Coulter counter, and aliquoted in defined medium. Defined medium consisted of DMEM supplemented with 5 µg/ml transferrin (Collaborative Research, Concord, MA), 10 µg/ml high density lipoprotein (HDL; Bionetics, Bethesda, MD), 1% BSA, and 20 mM HEPES.

#### Preparation of FN-Coated Substrata.

Human fibrinectin (FN) (Organon Teknika-Cappel, Malvern, PA) was coated (25 ng/well) in Lab-Tek 8-well culture slides (VWR) using 0.1 M

-26-

carbonate buffer, pH 9.4. Thirty minute incubations with 1% BSA/DMEM prior to plating cells blocked non-specific binding sites on the glass substrata.

5    Preparation of coated microbeads.

Tosyl-activated magnetic microbeads (4.5  $\mu$ m diameter; Dynal Inc.) were coated (all at 50  $\mu$ g/ml in 0.1 M carbonate buffer, pH 9.4; 22) with FN, AcLDL (Biomedical Technologies Inc.), or RGD-  
10    containing peptide (Peptide 2000; Telios Pharmaceuticals). After 18 hr, the round adherent cells were incubated with microbeads (10 beads/cell) for 15 or 30 min, washed in cold PBS, and placed on ice. The beads were then washed  
15    three times with 1% BSA/DMEM, and stored at 4°C in 1% BSA/DMEM at a final concentration of  $2 \times 10^7$  beads/ml.

Separation of CSK and basal cell surfaces.

To identify CSK-associated FAC proteins,  
20    cells were detergent-extracted for 1 min in ice cold CSK extraction buffer (CSK-EB; 0.5% Triton-X-100, 50 mM NaCl, 300 mM sucrose, 3 mM MgCl<sub>2</sub>, 20  $\mu$ g/ml aprotinin, 1  $\mu$ g/ml leupeptin, 1  $\mu$ g/ml pepstatin, 1 mM PMSF, 10 mM PIPES, pH 6.8) that  
25    maintains the integrity of the CSK, as described by Burr, et al., (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 3484-3488. The same method was used to isolate the whole CSK fraction from confluent monolayers for Western blot analysis. To isolate basal cell  
30    surfaces, nitrocellulose paper (0.45  $\mu$ m pore; Schleicher and Schuell) was wetted in water, placed on top of a cell monolayer for 15 sec, and then quickly removed with forceps, thereby "wet cleaving" the cells and removing the apical cell  
35    surfaces, as described by Brands, R. and Feltkamp, C.A. (1988) Exp. Cell Res. 176, 309-318. The remaining adherent basal cell surfaces were scraped

-27-

into PBS containing 1% aprotinin and pelleted by centrifugation.

#### Isolation of FACS.

Cells dispersed with trypsin-EDTA were washed twice in 1% BSA/DMEM, placed in polypropylene tubes (Costar), suspended ( $1 \times 10^6$  cells/ml) in defined medium containing magnetic RGD-beads ( $2 \times 10^7$ /ml), and rotated for 30 min at 37°C. RGD-coated beads were chosen for large-scale isolation of FACS because they exhibited less non-specific clumping during magnetic pelleting. Microbeads and bound cells were collected by placing a magnet at the side of the tubes, suspended in ice cold CSK-EB without detergent, and transferred to 5 ml polystyrene tubes (VWR). All subsequent procedures were carried out at 4°C using a side pull magnetic separation unit (Advanced Magnetix). The magnetic bead pellet was then transferred into complete CSK-EB, sonicated for 10 sec at output setting of 4, output power 10% (XL2005 Cell Disrupter, Heat Systems Ultrasonics), and homogenized in a 2 ml dounce homogenizer (20 strokes). The microbeads were magnetically pelleted and washed 5 times with CSK-EB prior to use;  $1 \times 10^7$  cells yielded 1 mg FAC protein.

#### Example 2: Characterization of Isolated FACS.

Chemical signaling molecules such as c-src, FAC kinase, phospholipase C, PI-3-kinase, and Na<sup>+</sup>/H<sup>+</sup> antiporter, were localized within FACS *in situ* by immunofluorescence and biochemically confirmed that they are enriched within isolated FACS *in vitro*.

#### MATERIALS AND METHODS

##### Immunofluorescence Microscopy.

A detergent permeabilization technique was used to identify proteins that became associated with the cytoskeleton and to increase sensitivity

-28-

of resolution of focal adhesion-associated proteins. Briefly, cells were extracted in ice cold cytoskeletal extraction buffer (CSK-EB) containing 10 mM PIPES, pH 6.8, 50 mM NaCl, 300 mM sucrose, 3 mM MgCl<sub>2</sub>, 20 µg/ml aprotinin (Boehringer-Mannheim), 1 µg/ml leupeptin (Boehringer Mannheim), 1 µg/ml pepstatin (Boehringer Mannheim), and 1 mM phenylmethylsulfonyl fluoride supplemented with 0.5% Triton X 100. Each well was then washed briefly with ice cold PBS and fixed at room temperature for 30 min in 4% paraformaldehyde (Electron Microscopy Sciences) in PBS.

#### Antibodies.

Rabbit antiserum against talin, paxillin, and α-actinin were provided by Dr. Keith Burridge (Univ. North Carolina, Chapel Hill). Rabbit antiserum against phosphotyrosine was a generous gift of Dr. Morris White (Joslin Diabetes Center, Boston, MA). Additional rabbit antiserum and mouse monoclonal antibodies raised against phosphotyrosine were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Affinity purified rabbit antibodies against actin were purchased from Biomedical Technologies, Inc. Rabbit antiserum against the β<sub>1</sub> chain of integrin was provided by Dr. Richard Hynes (Mass. Institute of Technology). Affinity purified rhodamine-conjugated antibodies were purchased from Organon Teknika-Cappel. Affinity purified peroxidase labelled antibodies were purchased from Amersham. Affinity purified rabbit antibodies against actin were purchased from Boehringer Mannheim. Affinity purified peroxidase labelled antibodies were purchased from Amersham.

Fixed cells with attached beads were exposed to primary antibodies diluted in IF buffer (0.2% Triton X100/0.1% Bovine serum albumin

-29-

(BSA)/PBS), washed 3X with PBS, then incubated for an additional hour in rhodamine-conjugated secondary antibodies (Goat anti-Rabbit IgG or Goat anti-mouse IgG) diluted 1:250 in IF buffer.

5                Photographic images were recorded on Kodak Tri-X 400 film using a Zeis Photo II microscope equipped for epifluorescence.

              Past studies show that FN-coated magnetic microbeads bind tightly to endothelial cells and  
10    activate intracellular chemical signaling pathways (e.g.,  $\text{Na}^+/\text{H}^+$  antiporter) within 10 to 30 min following bead binding (Schwartz et al., Proc. Natl. Acad. Sci. USA 88:7849-7853 (1991)). These beads were used in conjunction with  
15    immunofluorescence microscopy to analyze FAC assembly at similar early times (Schwartz et al., 1991). The FAC proteins, integrin  $\beta_1$ , talin, vinculin, paxillin, and  $\alpha$ -actinin were recruited to the cell surface directly beneath the site of bead  
20    binding within 15 min after bead addition, as shown by Figure 3. FAC formation first appeared in a crescent along the bead-cell surface interface which then progressed to encircle the entire bead surface by approximately 30 min. FAC formation was  
25    also accompanied by increased cortical actin staining in the region of the bead. In contrast, beads coated with AcLDL, which binds to transmembrane "scavenger" receptors, did not induce recruitment of any FAC protein although increased  
30    cortical actin staining was also observed. This rapid appearance of actin under both conditions may represent a change in the distribution of microfilaments due to mechanical deformation of the cell cortex rather than actual recruitment of new  
35    protein to the FAC. Similar staining patterns were obtained using RGD-beads and neither bead exhibited staining for myosin, ankyrin, or tubulin.

-30-

Importantly, use of microbeads coated with integrin ligands also permitted morphological analysis of the process of FAC assembly. For example, quantitation of bound beads that exhibited staining at 15 and 30 min revealed that different proteins were recruited to the bead-associated FAC at different rates (Figure 3: actin = integrin  $\beta_1$   $\geq$  talin  $>$   $\alpha$ -actinin  $\geq$  paxillin  $>>$  vinculin). The slow recruitment of vinculin may explain why this FAC component was not detected in previous immunolocalization studies using ECM-coated microbeads, reported by Mueller, et al., (1989) J. Cell Biol. 109, 3455-3464 and Grinnell, F. and Geiger, B. (1986) Exp. Cell Res. 162, 449-461.

15        Isolation of the FAC.

The magnetic properties of the microbeads also provides a means to physically isolate these newly formed FACs from the remainder of the cell. After incubation with RGD-beads in suspension for 30 min, cells with bound beads were magnetically pelleted, detergent extracted in a buffer that maintains CSK integrity, sonicated to physically disrupt the CSK, dounce homogenized to remove nuclei, and extensively washed to remove cellular structures not intimately associated with the beads. Light microscopic analysis confirmed that this procedure resulted in isolation of individual beads which lacked any visible cellular extensions.

25        Western Blot Analysis.

30        Proteins in the bead complexes, whole CSK pellet, and basal membrane preparations were dissolved in RIPA buffer (1% Triton X-100, 1% deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM Tris pH 7.2, 1% PMSF), separated by SDS-PAGE (7.5% acrylamide), and transferred to nitrocellulose (0.45  $\mu$ m pore) using a semi-dry blotting system (Polyblot SBD-1000; American Bionetics). Equal

35



-31-

protein (50  $\mu$ g), as determined using a BCA protein microassay (Pierce), was loaded in each gel lane. Non-specific binding sites on the nitrocellulose were blocked with 0.5% casein in TBS and incubated sequentially with primary rabbit antibodies (all diluted 1:500 in TBS containing 0.05% Tween 20) and affinity-purified horseradish peroxidase-conjugated goat anti-rabbit immunoglobulins (1:6000 dilution). Labelled proteins were visualized using an ECL chemiluminescence kit (Amersham). Densitometric quantitation was carried out using a Datacopy GS Plus optical scanner and NIH public domain Image software (Masters, et al., Biotechniques 12, 902-911 (1992)).

As shown by Figures 4 and 5, western blotting revealed that all of the FAC components that were visualized along the cell-bead interface in living cells were retained in the isolated bead complexes. Isolated FACs were greatly enriched for integrin  $\beta_1$ , vinculin, paxillin, talin, and  $\alpha$ -actinin when compared with either extracts of the whole CSK or a crude preparation of basal cell membranes. In contrast, the bead complexes were not enriched for actin. Densitometric quantitation of these results revealed that vinculin, paxillin, talin, and  $\alpha$ -actinin were concentrated approximately 53, 11, 1.5 and 2 fold, respectively, in the isolated FAC relative to the whole CSK and 5, 7, 2 and 10 fold relative to basal cell membranes. Under these conditions in which equal protein was loaded, integrin  $\beta_1$  could not be visualized in the whole CSK fraction although it was a major component of the isolated FAC. Furthermore, the efficiency of the isolation method was also much greater than that obtained using past approaches (Avnur, Z. and Geiger, B. (1981) J. Mol. Biol. 153, 361-379; Brands, R. and Feltkamp, C.A.

-32-

(1988) Exp. Cell Res. 176, 309-318; and Gates, et al., (1993) Biochem. J. 289, 221-226). As an example, integrin  $\beta_1$  was enriched over 50 fold in the FAC compared to the basal cell membrane fraction obtained by the wet-cleaved method. On the other hand, approximately twice the amount of actin was found in the whole CSK fraction compared with the isolated FAC, confirming that the intact FAC was physically removed from the remainder of the CSK during magnetic isolation. Protein complexes that were isolated from cells bound to AcLDL beads using identical separation methods in the same experiment did not contain sufficient protein for biochemical analysis.

15        Two dimensional gels

Proteins isolated in the FAC are also analyzed by two-dimensional PAGE using the method of O'Farrell, P.H., J. Biol. Chem. 250: 4007-4021 (1975) by Kendrick Labs, Inc. (Madison, WI) as follows: Isoelectric focusing is carried out in glass tubes of inner diameter 2.0 mm, using 2% BDH pH 4-8 ampholines, for 9600 volt-hrs. The final tube gel pH gradient as measured by a surface pH electrode is on the enclosed pH gradient form.

25        After equilibration for 10 min in Buffer 'O' (10% glycerol, 50 mM dithiothreitol, 2.3% SDS and 0.0625 M tris, pH 6.8), the tube gel is sealed to the top of a stacking gel on the top of a 10% acrylamide slab gel (0.75 mm thick) and SDS slab gel electrophoresis carried out for about 4 hrs at 12.5 mA/gel. The slab gel is stopped when the bromophenol dye front reached the bottom of the gel. After slab gel electrophoresis the slab gel is transferred to transfer buffer (12.5 mM TRIS, pH 30 8.8, 86 mM Glycine, 10% MeOH) transblotted onto Problot paper overnight at room temperature, at 145 35 mA and approximately 50 volts/gel.

-33-

Gels were loaded with equal amounts of proteins (20  $\mu$ g) from the isolated FAC fraction or the whole cytoskeleton (CSK) and stained with india ink. Figure 6a is a gel of the FAC, with proteins not found in whole CSK circled. Figure 6b is a gel of the whole CSK.

In summary, the data shows that binding of microbeads coated with integrin ligands rapidly induces formation of intact FACs containing integrin  $\beta_1$ , talin, vinculin, paxillin, and  $\alpha$ -actinin at the bead-cell interface in round cells. It also shows that these magnetic microbeads can be used to isolate intact FACs in sufficient quantity to allow biochemical characterization of their constituent proteins. Furthermore, the isolated FACs exhibit a molecular composition similar to that observed *in situ*.

Importantly, this method provides many advantages when compared to those used in the past to enrich for FAC proteins: (1) the isolated FAC is physically separated from contaminating cell surface proteins and the remainder of the actin CSK, (2) the efficiency of FAC protein enrichment is much greater, (3) FAC assembly can be separated from the CSK changes that accompany cell spreading, and (4) newly forming FACs can be isolated within minutes after integrin ligation. This last advantage is perhaps most critical. Most past studies analyzed FACs many hours after they have formed and second messengers have been released. In contrast, this method offers a unique opportunity to study and isolate FACs at a time when molecular linkages between ECM and the CSK are just forming and integrin signaling pathways are first activated (Ingber (1990), Schwartz (1991), Kornberg, et al., (1991) Proc. Natl. Acad. Sci. U.S.A. 88, 8392-8396; and Guan, J.L. and Shalloway,

-34-

D. (1992) Nature 358:690-692). Thus, this simple method may be used to examine the sequential biochemical changes that lead to FAC assembly and to analyze FAC structure-function relations at critical times during cell activation *in situ* and after isolation under defined conditions *in vitro*.

Example 3: Functional Characterization of FAC isolated in Example 1.

The isolated FAC also retained functional protein and lipid kinase activity similar to that observed in intact cells, as demonstrated below.

MATERIALS AND METHODS

Protein kinase assay.

Isolated RGD- or Ac-LDL-bead protein complexes were washed with kinase buffer (10 mM Tris, pH 7.2/4.9 mM MgCl<sub>2</sub>/200  $\mu$ M NaVO<sub>4</sub>) then incubated with 100  $\mu$ Ci/ml gamma-<sup>32</sup>P-ATP (New England Nuclear) at 37°C for 15 minutes. The bead complexes were washed 10 x with kinase buffer, then boiled in SDS-PAGE sample buffer (Laemmli) to remove them from the beads. Equal CPM from each sample were electrophoresed on 7.5% SDS-polyacrylamide gels. The gels were fixed in 20% MeOH/10% acetic acid, treated with Resolution autoradiography enhancer (EM Corp., Chestnut Hill, MA), dried, and exposed to film. Films were developed using a Fugii RGII X-ray film processor.

SDS-PAGE and Analysis of Proteins by Function.

GTP binding proteins are also present in FACs, as demonstrated by separation of the FAC proteins on a 12% SDS PAGE gel, blotting onto nitrocellulose, and determining GTP binding activity by incubating with a <sup>32</sup>P-GTP, followed by ARG. Lane 1 was incubated with a <sup>32</sup>P-GTP alone, lanes 2 and 3 were pre-incubated with 50 nM GTP or ATP, respectively, to determine whether the binding was specific for GTP, according to the method of

-35-

Lapetina and Reep, "Specific binding of [ $\alpha$ - $^{32}$ P]GTP to cytosolic and membrane-bound proteins of human platelets correlates with the activation of phospholipase C" Proc. Natl. Acad. Sci. USA 84, 2261-2265 (1987).

Three major GTP-binding proteins appear which exhibit mobilities consistent with molecular weights of approximately 22,000, 24,000 and 25,000 daltons. The results show GTP-specific binding by these proteins.

PIP kinase activity is also present in FAC and is enriched relative to preparations of cytoskeleton or basolateral cell membranes. Protein samples were incubated with liposomes containing PIP and PS in the presence of gamma- $^{32}$ P-ATP. Labelled lipids were extracted with chloroform, separated by TLC and visualized by ARG. In the presence of PIP kinase and gamma- $^{32}$ P-ATP, unlabelled PIP is phosphorylated to labelled PIP<sub>2</sub>, as reported by Ling, et al., J. Biol. Chem. 264, 5080-5088 (1989). When isolated FACs were incubated under these conditions, production of labelled PIP<sub>2</sub> was observed. In addition, a labelled PIP spot was observed when gamma- $^{32}$ P-ATP was added in the absence of exogenous lipid substrates, indicating that endogenous PI is isolated by this method and retained within the FAC in a form accessible to PI kinase.

The latter result shows that both a specific lipid kinase (PI kinase) and its specific membrane lipid substrate (PI) remain physically associated and in close and physiologically relevant proximity to the demembranated FAC complex. Furthermore, high levels of both PIP and PIP<sub>2</sub> synthesis can be demonstrated when exogenous unlabelled substrates (PI and PIP) are added to the isolated FACs in the presence of  $^{32}$ P-ATP. This

-36-

means that functional PI and PIP kinases are both retained within the isolated FAC.

The endogenous protein kinase activity in the isolated complexes was assayed by incubating them with [ $\gamma$ - $^{32}$ P] ATP. When compared to whole cell cytoskeleton and complexes from Ac-LDL beads, phosphorylation of specific protein bands in isolated FACs was observed. In addition, the phosphorylation pattern produced in isolated FACs closely resembles that produced when FACs are isolated from cells prelabelled with  $^{32}$ P. These results demonstrate that specific protein kinases remain functional within the FAC even after isolation.

Example 4: Use of isolated FACs to analyze the effects of ECMs, Integrins, and other compounds affecting the mechanical and chemical signaling pathways.

The methods described in the above examples have been used to demonstrate that the compound herbimycin A, which inhibits angiogenesis of capillary cells by inhibition of capillary endothelial cell growth at 0.5  $\mu$ g/ml, also inhibits tyrosine phosphorylation, 15 to 30 minutes after binding. This was determined by finding that herbimycin A suppressed tyrosine phosphorylation within the FAC by both immunofluorescence staining (*in situ*) and biochemical analysis (*in vitro*), that it did not alter the recruitment of c-src to the FAC, and that it inhibited the phosphorylation of one single protein species with a molecular weight of 60 kDa, which is the size of multiple members of the src tyrosine kinase family. Accordingly, the screening method described herein has demonstrated that this angiogenesis inhibitor modulates the function of a specific chemical signalling molecule that is activated by integrin ligation and physically contained within the FAC.

-37-

These methods allow one to specifically confirm the molecular identity of the target protein and, at the same time, use the identical assays to screen through chemical libraries to  
5 identify related chemical analogues as well as unrelated compounds to identify even more potent angiogenesis inhibitors.

Mechanical signaling modulators can also be identified by screening for proteins that either  
10 disrupt FAC integrity or specifically inhibit mechanical force transfer across the cell surface and to the cytoskeleton. The former function can be identified by radiolabelling the FAC proteins on the beads (e.g., isolated FACs from <sup>35</sup>S-methionine-  
15 labelled cells) and then identify exogenous compounds that cause the FACs to dissolved under physiological conditions (e.g., quantitating radioactive counts that are released into the supernatant when the beads are mechanically  
20 pelleted). The latter function can be screened using a magnetic probe which applies mechanical forces directly to integrins using applied magnetic fields and at the same time measures the cytoskeletal response to force, as described by  
25 Wang, et al., Mol. Cell Biol. September 1992. Using this system, integrins have been shown to transfer force to the cytoskeleton and support a cytoskeletal stiffening response whereas non-adhesion receptors, such as AcLDL receptors, do  
30 not. Thus, modulators that bind FACs or modulate chemical signaling in the FACs can be tested directly for their ability to alter mechanical signalling.

For example, these methods could be used to  
35 identify the target as c-src or related src-like protein kinases, providing the incentive to develop antibodies against the protein, or clone the gene

-38-

encoding the protein, which can be used to make peptides or antisense against the gene. These functional modulators are then used to inhibit the particular pathway in the intact cell.

5           One of the most fundamental changes in transformed cells is that they grow when round and free of anchorage whereas normal cells must attach and spread in order to respond to soluble growth factors and proliferate. Recent studies have  
10           revealed that these cell shape changes that are required for growth result from mechanical interactions within a continuous and globally integrated CSK. Mechanical tension is generated within intracellular contractile microfilaments via  
15           an actomyosin filament sliding mechanism, transmitted across transmembrane "integrin" receptors, and resisted by external anchoring sites within the extracellular matrix. Altering this force balance results in coordinated changes in  
20           cell, CSK, and nuclear structure, as reported by Sims, et al., J. Cell Sci. 103, 1215-1222 (1982). Importantly, loss of structural continuity between the CSK and integrins, disruption of transmembrane force transmission, and uncoupling between cell  
25           shape and growth all accompany neoplastic transformation.

          Given that integrin receptors physically interconnect the CSK with the extracellular matrix, they present a window on the CSK, and can be used  
30           as a basis for a technique to quantitate changes in mechanical signaling within FACS of living cells. Ferromagnetic microbeads, 5  $\mu$ m diameter, are coated with specific integrin receptor ligands and allowed to bind to the surface of adherent cells cultured  
35           in a single microwell. By applying a brief but intense magnetic pulse, the beads are magnetized in one direction; by subsequently applying a weaker



-39-

magnetic field oriented at 90° to the initial orientation, the bead is exposed to a magnetic torque and twists in response. A sensitive in-line magnetometer measures changes in the remanent field vector in the direction of the original magnetization and, hence, the degree of bead rotation. Thus, both torque (shear stress) applied to specific cell surface integrin receptors and the resulting bead rotation are measured. From these relative simple measurements specific mechanical properties of the CSK (shear stiffness, elastic recoil, apparent viscosity) and their changes can be measured in real-time in living cells.

In studies using this device, it has been possible to show that stress-induced changes in cytomechanics result from global structural rearrangements of the CSK. Specific changes in CSK mechanics also were observed when the shape of the cell was altered. Since this non-invasive technique probes the very structure of the cell, it allows identification of specific cytomechanical "signatures" that are prognostic of changes in cell growth as well as neoplastic transformation.

It has been found that the alterations in CSK mechanics measured were predictive of changes in proliferation in normal (non-transformed) cells. In contrast, tumor cells exhibited both different mechanical properties (e.g., they were much less stiff) and deregulated growth under similar culture conditions.

This technology can be used to identify new anti-cancer compounds based on their ability to modulate mechanical signalling across the FAC to produce a specific cytomechanical response. Measuring changes in the CSK of cells from tumor biopsies also provides a rapid method to characterize tumor cell sensitivity to currently

-40-

available anti-cancer drugs. For example, a potent angiogenesis inhibitor (TNP-470) that is currently in Phase I clinical trials for the treatment of solid tumors produces a specific change in CSK mechanics in its target, the capillary endothelial cell. The shear stiffness of the CSK increases by 30% within 15 min after drug addition. Another anti-cancer agent, Taxol, also increases CSK stiffness, but only by 10%. Interestingly, Taxol was only about half as effective as TNP-470 at inhibiting the growth of these cells. Isolated complexes from TNP-470 treated cells exhibit three distinct phosphotyrosine containing protein bands which either increase or decrease in a time-dependent manner within minutes after drug application. At the same time and dose, TNP-470 induces a dramatic increase in cell stiffness measured using the magnetic twisting device whereas a closely related chemical analogue that is inactive, i.e., does not inhibit capillary growth or angiogenesis, failed to produce this response.

This magnetic probe technology is a simple and rapid method for screening of compounds that alter mechanical signaling between extracellular matrix and the cytoskeleton by modulating FAC structure or function, and hence, which is useful for control of normal and tumor cell growth and function. The changes in FAC phosphorylation and cell mechanics provide, for the first time, measurable events that can form the basis of a rapid drug screening assay, especially for angiogenesis inhibitors.

Modifications and variations of the present invention will be obvious to those skilled in the art. Such modifications and variations are intended to come within the scope of the appended claims.

-41-

We claim:

1. A method to isolate intact focal adhesion complexes comprising:

mixing cells with microbeads coated with a molecule that ligates integrins, even through receptors other than integrins, and thereby mediates attachment and spreading and induces formation of the functionally active FACS;

mechanically agitating the mixture until the microbeads are bound to the cells and focal adhesion complexes have formed at the binding sites between the microbeads and the cells;

extracting the microbead-bound cells with a non-ionic detergent to remove membranes and soluble proteins from the microbeads while maintaining the structural integrity of the actin cytoskeleton;

sonicating the microbeads to disrupt the remaining cytoskeleton bound to the microbeads;

homogenizing the microbeads to remove residual large pieces of cytoskeletal-nuclear matrix lattice and nuclei bound to the microbeads; and

collecting the microbeads.

2. The method of claim 1 wherein the integrin ligand is selected from the group consisting of proteins and peptides characterized by inclusion of attachment sites and portions and derivatives thereof containing an attachment site, toxins binding to integrins, antibodies to integrins, antibodies to heparan sulfate, heparin-binding peptides, synthetic peptides binding to heparin, growth factors binding to heparin, viral proteins binding to heparin, Sugars which bind to cell surface glycosyltransferase, peptides binding to laminin receptors), and receptor-specific antibodies.

-42-

3. The method of claim 1 wherein the microbead has a diameter between 0.1 and 50 microns in diameter.

4. The method of claim 1 wherein the microbead is formed of a magnetic or magnetizable material.

5. The method of claim 1 further comprising removing the focal adhesion complexes from the microbeads to yield isolated focal adhesion complexes.

6. The method of claim 5 further comprising separating the components of the focal adhesion complexes.

7. The method of claim 6 further comprising removing the known proteins from the unknown proteins in the focal adhesion complexes.

8. The method of claim 7 wherein the proteins are separated by gel electrophoresis.

9. The method of claim 8 further comprising cloning the DNA molecules encoding the separated unknown proteins.

10. The method of claim 8 further comprising making antibodies to the separated unknown proteins.

11. The method of claim 8 further comprising sequencing the separated unknown proteins.

12. The method of claim 11 further comprising making DNA probes to the DNA molecules encoding the separated unknown proteins.

13. The method of claim 11 further comprising making peptides from the separated unknown proteins.

14. An isolated, functionally active focal adhesion complex.

-43-

15. The complex of claim 14 isolated by mixing cells with microbeads coated with a molecule that ligates integrins, even through receptors other than integrins, and thereby mediates attachment and spreading and induces formation of the functionally active FACS;

mechanically agitating the mixture until the microbeads are bound to the cells and focal adhesion complexes have formed at the binding sites between the microbeads and the cells;

extracting the microbead-bound cells with a non-ionic detergent to remove membranes and soluble proteins from the microbeads while maintaining the structural integrity of the actin cytoskeleton;

sonicating the microbeads to disrupt the remaining cytoskeleton bound to the microbeads;

homogenizing the microbeads to remove residual large pieces of cytoskeletal-nuclear matrix lattice and nuclei bound to the microbeads; and

collecting the microbeads.

16. The method of claim 15 wherein the integrin ligand is selected from the group consisting of proteins and peptides characterized by inclusion of attachment sites and portions and derivatives thereof containing an attachment site, toxins binding to integrins, antibodies to integrins, antibodies to heparan sulfate, heparin-binding peptides, synthetic peptides binding to heparin, growth factors binding to heparin, viral proteins binding to heparin, sugars which bind to cell surface glycosyltransferase, peptides binding to laminin receptors), and receptor-specific antibodies.

17. The method of claim 16 further comprising separating the focal adhesion complexes from the microbeads.

-44-

18. The unknown proteins isolated by mixing cells with microbeads coated with a molecule that ligates integrins, even through receptors other than integrins, and thereby mediates attachment and spreading and induces formation of the functionally active FACS;

mechanically agitating the mixture until the microbeads are bound to the cells and focal adhesion complexes have formed at the binding sites between the microbeads and the cells;

extracting the microbead-bound cells with a non-ionic detergent to remove membranes and soluble proteins from the microbeads while maintaining the structural integrity of the actin cytoskeleton;

sonicating the microbeads to disrupt the remaining cytoskeleton bound to the microbeads;

homogenizing the microbeads to remove residual large pieces of cytoskeletal-nuclear matrix lattice and nuclei bound to the microbeads;

collecting the microbeads;

removing the focal adhesion complexes from the microbeads;

separating the components in the focal adhesion complexes; and

separating the known proteins from the unknown proteins in the focal adhesion complexes by gel electrophoresis.

19. An antibody to an unknown protein isolated by

mixing cells with microbeads coated with a molecule that ligates integrins, even through receptors other than integrins, and thereby mediates attachment and spreading and induces formation of the functionally active FACS;

mechanically agitating the mixture until the microbeads are bound to the cells and focal

-45-

adhesion complexes have formed at the binding sites between the microbeads and the cells;

extracting the microbead-bound cells with a non-ionic detergent to remove membranes and soluble proteins from the microbeads while maintaining the structural integrity of the actin cytoskeleton;

sonicating the microbeads to disrupt the remaining cytoskeleton bound to the microbeads;

homogenizing the microbeads to remove residual large pieces of cytoskeletal-nuclear matrix lattice and nuclei bound to the microbeads;

collecting the microbeads;

removing the focal adhesion complexes from the microbeads;

separating the components in the focal adhesion complexes; and

separating the known proteins from the unknown proteins in the focal adhesion complexes by gel electrophoresis.

20. A nucleic acid probe obtained by mixing cells with microbeads coated with a molecule that ligates integrins, even through receptors other than integrins, and thereby mediates attachment and spreading and induces formation of the functionally active FACS;

mechanically agitating the mixture until the microbeads are bound to the cells and focal adhesion complexes have formed at the binding sites between the microbeads and the cells;

extracting the microbead-bound cells with a non-ionic detergent to remove membranes and soluble proteins from the microbeads while maintaining the structural integrity of the actin cytoskeleton;

sonicating the microbeads to disrupt the remaining cytoskeleton bound to the microbeads;

-46-

homogenizing the microbeads to remove residual large pieces of cytoskeletal-nuclear matrix lattice and nuclei bound to the microbeads;  
collecting the microbeads;  
removing the focal adhesion complexes from the microbeads;  
separating the components in the focal adhesion complexes;  
separating the known proteins from the unknown proteins in the focal adhesion complexes by gel electrophoresis; and  
amino acid sequencing the isolated protein.

21. The method of claim 20 further comprising making nucleic acid primers from the amino acid sequence and isolating the DNA encoding the unknown proteins.

22. The method of claim 20 further comprising making peptides binding to FAC proteins from the amino acid sequence of the isolated protein.

23. A method for screening for a compound having an effect on focal adhesion complexes comprising:

exposing cells to the compound to be screened;  
isolating the focal adhesion complexes; and  
determining the effect of the compound on the focal adhesion complex or components thereof.

24. The method of claim 23 wherein the focal adhesion complexes are obtained by

mixing cells with microbeads coated with a molecule that ligates integrins, even through receptors other than integrins, and thereby mediates attachment and spreading and induces formation of the functionally active FACs;

mechanically agitating the mixture until the microbeads are bound to the cells and focal



-47-

adhesion complexes have formed at the binding sites between the microbeads and the cells;

extracting the microbead-bound cells with a non-ionic detergent to remove membranes and soluble proteins from the microbeads while maintaining the structural integrity of the actin cytoskeleton;

sonicating the microbeads to disrupt the remaining cytoskeleton bound to the microbeads;

homogenizing the microbeads to remove residual large pieces of cytoskeletal-nuclear matrix lattice and nuclei bound to the microbeads; and

collecting the microbeads.

25. The method of claim 24 wherein the receptor ligand is selected from the group consisting of proteins and peptides characterized by inclusion of attachment sites and portions and derivatives thereof containing an attachment site, toxins binding to integrins, antibodies to integrins, antibodies to heparan sulfate, heparin-binding peptides, synthetic peptides binding to heparin, growth factors binding to heparin, viral proteins binding to heparin, sugars which bind to cell surface glycosyltransferase, peptides binding to laminin receptors), and receptor-specific antibodies.

26. The method of claim 25 where the focal adhesion complexes are removed from the microbeads.

27. The method of claim 24 where the compound is exposed to the isolated focal adhesion complex and the effect determined.

28. The method of claim 24 where the compound is exposed to cells, the focal adhesion complex is formed between the cells and the microbeads, and the effect on the isolated focal adhesion complex of the compound determined.

## AMENDED CLAIMS

[received by the International Bureau on 27 September 1994 (27.09.94);  
original claims 1-28 replaced by amended claims 1-28 (8 pages)]

1. A method to isolate intact focal adhesion complexes containing multiple interconnected functionally interactive molecules present at sites beneath the cell membrane where the cell cytoskeleton binds via transmembrane receptors to extracellular ligands, substantially free of other cellular components, comprising:

mixing cells with microbeads coated with a molecule that ligates transmembrane receptors that connect to the cytoskeleton and thereby mediates attachment and spreading and induces formation of the functionally active FACS;

mechanically agitating the mixture until the microbeads are bound to the cells and focal adhesion complexes have formed at the binding sites between the microbeads and the cells;

extracting the microbead-bound cells with a non-ionic detergent to remove membranes and soluble proteins from the microbeads while maintaining the structural integrity of the actin cytoskeleton;

sonicating the microbeads to disrupt the remaining cytoskeleton bound to the microbeads;

homogenizing the microbeads to remove residual large pieces of cytoskeletal-nuclear matrix lattice and nuclei bound to the microbeads; and

collecting the microbeads.

2. The method of claim 1 wherein the ligand is selected from the group consisting of proteins and peptides characterized by inclusion of attachment sites and portions and derivatives thereof containing an attachment site, toxins binding to integrins, antibodies to integrins, antibodies to heparan sulfate, heparin-binding peptides, synthetic peptides binding to heparin, growth factors binding to heparin, viral proteins binding to heparin, sugars which bind to cell surface glycosyltransferase, peptides binding to laminin receptors, and receptor-specific antibodies.

3. The method of claim 1 wherein the microbead has a diameter between 0.1 and 50 microns in diameter.

4. The method of claim 1 wherein the microbead is formed of a magnetic or magnetizable material.

5. The method of claim 1 further comprising removing the focal adhesion complexes from the microbeads to yield isolated focal adhesion complexes.

6. The method of claim 5 further comprising separating the components of the focal adhesion complexes.

7. The method of claim 6 further comprising identifying proteins within the complex and removing the identified proteins from the unidentified proteins in the focal adhesion complexes.

8. The method of claim 7 wherein the proteins are separated by gel electrophoresis.

9. The method of claim 8 further comprising cloning the DNA molecules encoding the separated proteins.

10. The method of claim 8 further comprising making antibodies to the separated proteins.

11. The method of claim 8 further comprising sequencing the separated proteins.

12. The method of claim 11 further comprising making DNA probes to the DNA molecules encoding the separated proteins.

13. The method of claim 11 further comprising making peptides from the separated proteins.

14. An isolated, functionally active focal adhesion complex containing multiple interconnected functionally interactive molecules present at sites beneath the cell membrane where the cell cytoskeleton binds via transmembrane receptors to extracellular ligands, substantially free of other cellular components.

15. The complex of claim 14 isolated by mixing cells with microbeads coated with a molecule that ligates transmembrane receptors that connect to the cytoskeleton, and thereby mediates attachment and spreading and induces formation of the functionally active FACS; mechanically agitating the mixture until the microbeads are bound to the cells and focal adhesion complexes have formed at the binding sites between the microbeads and the cells; extracting the microbead-bound cells with a non-ionic detergent to remove membranes and soluble proteins from the microbeads while maintaining the structural integrity of the actin cytoskeleton; sonicating the microbeads to disrupt the remaining cytoskeleton bound to the microbeads; homogenizing the microbeads to remove residual large pieces of cytoskeletal-nuclear matrix lattice and nuclei bound to the microbeads; and collecting the microbeads.

16. The complex of claim 15 wherein the ligand used in the method is selected from the group consisting of proteins and peptides characterized by inclusion of attachment sites and portions and derivatives thereof containing an attachment site, toxins binding to integrins, antibodies to integrins, antibodies to heparan sulfate, heparin-binding peptides, synthetic peptides binding to heparin, growth factors binding to heparin, viral proteins binding to heparin, sugars which bind to cell surface glycosyltransferase, peptides binding to laminin receptors, and receptor-specific antibodies.

17. The method of claim 16 further comprising separating the focal adhesion complexes from the microbeads.

18. A protein isolated from focal adhesion complexes containing multiple interconnected functionally interactive molecules present at sites beneath the cell membrane where the cell cytoskeleton binds via transmembrane receptors to extracellular ligands, substantially free of other cell components, by

mixing cells with microbeads coated with a molecule that ligates transmembrane receptors that connect to the cytoskeleton, and thereby mediates attachment and spreading and induces formation of the functionally active FACS;

mechanically agitating the mixture until the microbeads are bound to the cells and focal adhesion complexes have formed at the binding sites between the microbeads and the cells;

extracting the microbead-bound cells with a non-ionic detergent to remove membranes and soluble proteins from the microbeads while maintaining the structural integrity of the actin cytoskeleton;

sonicating the microbeads to disrupt the remaining cytoskeleton bound to the microbeads;

homogenizing the microbeads to remove residual large pieces of cytoskeletal-nuclear matrix lattice and nuclei bound to the microbeads;

collecting the microbeads;

removing the focal adhesion complexes from the microbeads;

separating the components in the focal adhesion complexes; and

separating the known proteins from the unknown proteins in the focal adhesion complexes by gel electrophoresis.

19. An antibody to a protein present in focal adhesion complexes containing multiple interconnected functionally interactive molecules present at sites beneath the cell membrane where the cell cytoskeleton binds via transmembrane receptors to extracellular ligands, substantially free of other cellular components, isolated by

mixing cells with microbeads coated with a molecule that ligates transmembrane receptors that connect to the cytoskeleton, and thereby mediates attachment and spreading and induces formation of the functionally active FACS;

mechanically agitating the mixture until the microbeads are bound to the cells and focal adhesion complexes have formed at the binding sites between the microbeads and the cells;

extracting the microbead-bound cells with a non-ionic detergent to remove membranes and soluble proteins from the microbeads while maintaining the structural integrity of the actin cytoskeleton;

sonicating the microbeads to disrupt the remaining cytoskeleton bound to the microbeads;

homogenizing the microbeads to remove residual large pieces of cytoskeletal-nuclear matrix lattice and nuclei bound to the microbeads;

collecting the microbeads;

removing the focal adhesion complexes from the microbeads;

separating the components in the focal adhesion complexes; and

separating the known proteins from the unknown proteins in the focal adhesion complexes by gel electrophoresis.

## 53

20. A nucleic acid probe obtained by  
mixing cells with microbeads coated with a molecule  
that ligates transmembrane receptors that connect to the  
cytoskeleton, and thereby mediates attachment and spreading  
and induces formation of the functionally active FACs;  
mechanically agitating the mixture until the  
microbeads are bound to the cells and focal adhesion complexes  
have formed at the binding sites between the microbeads and  
the cells;  
extracting the microbead-bound cells with a non-  
ionic detergent to remove membranes and soluble proteins from  
the microbeads while maintaining the structural integrity of  
the actin cytoskeleton;  
sonicating the microbeads to disrupt the remaining  
cytoskeleton bound to the microbeads;  
homogenizing the microbeads to remove residual large  
pieces of cytoskeletal-nuclear matrix lattice and nuclei bound  
to the microbeads;  
collecting the microbeads;  
removing the focal adhesion complexes from the  
microbeads;  
separating the components in the focal adhesion  
complexes;  
separating identifiable proteins from unidentifiable  
proteins in the focal adhesion complexes by gel  
electrophoresis; and  
amino acid sequencing the isolated unidentifiable  
protein.

21. The method of claim 20 further comprising  
making nucleic acid primers from the amino acid sequence and  
isolating the DNA encoding the isolated unidentifiable  
proteins.

22. The method of claim 20 further comprising  
making peptides binding to FAC proteins from the amino acid  
sequence of the isolated protein.

## 5.4

23. A method for screening for a compound having an effect on focal adhesion complexes containing multiple interconnected functionally interactive molecules present at sites beneath the cell membrane where the cell cytoskeleton binds via transmembrane receptors to extracellular ligands comprising:

- exposing cells to the compound to be screened;
- isolating the focal adhesion complexes; and
- determining the effect of the compound on the focal adhesion complex or components thereof.

24. The method of claim 23 wherein the focal adhesion complexes are obtained by

- mixing cells with microbeads coated with a molecule that ligates integrins, even through receptors other than integrins, and thereby mediates attachment and spreading and induces formation of the functionally active FACs;

- mechanically agitating the mixture until the microbeads are bound to the cells and focal adhesion complexes have formed at the binding sites between the microbeads and the cells;

- extracting the microbead-bound cells with a non-ionic detergent to remove membranes and soluble proteins from the microbeads while maintaining the structural integrity of the actin cytoskeleton;

- sonicating the microbeads to disrupt the remaining cytoskeleton bound to the microbeads;

- homogenizing the microbeads to remove residual large pieces of cytoskeletal-nuclear matrix lattice and nuclei bound to the microbeads; and

- collecting the microbeads.



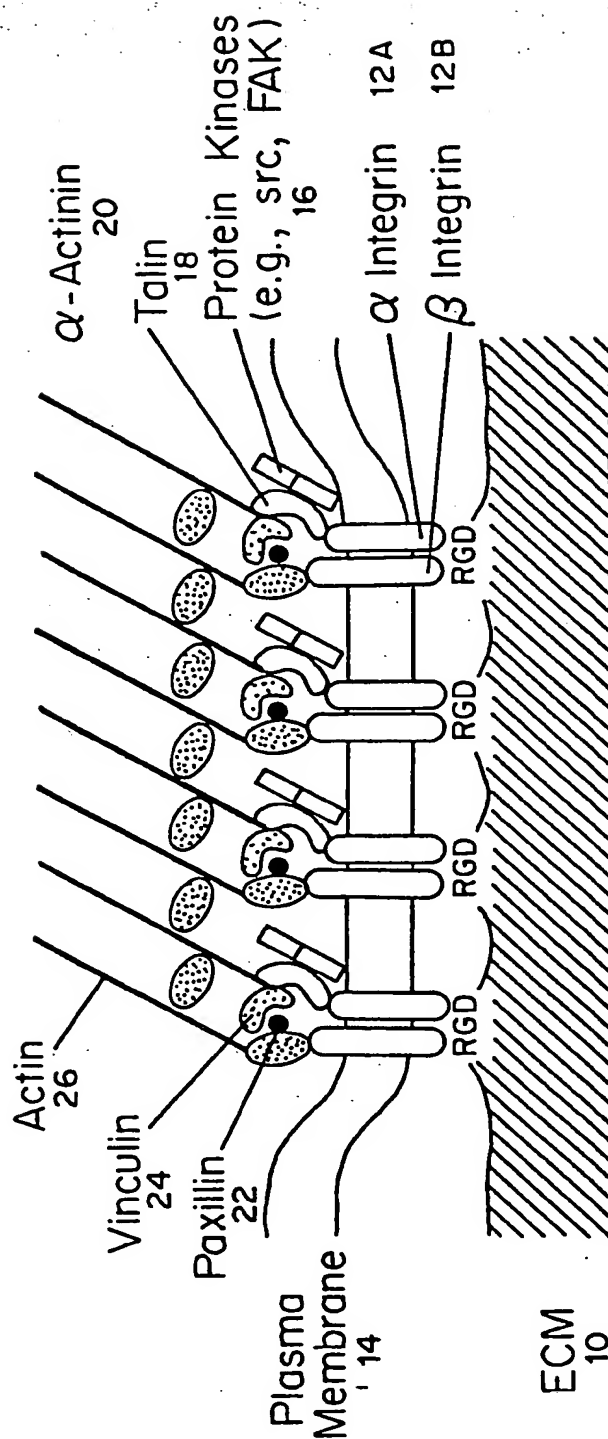
25. The method of claim 24 wherein the receptor ligand is selected from the group consisting of proteins and peptides characterized by inclusion of attachment sites and portions and derivatives thereof containing an attachment site, toxins binding to integrins, antibodies to integrins, antibodies to heparan sulfate, heparin-binding peptides, synthetic peptides binding to heparin, growth factors binding to heparin, viral proteins binding to heparin, sugars which bind to cell surface glycosyltransferase, peptides binding to laminin receptors), and receptor-specific antibodies.

26. The method of claim 25 where the focal adhesion complexes are removed from the microbeads.

27. The method of claim 24 where the compound is exposed to the isolated focal adhesion complex and the effect determined.

28. The method of claim 24 where the compound is exposed to cells, the focal adhesion complex is formed between the cells and the microbeads, and the effect on the isolated focal adhesion complex of the compound determined.

- 1 / 6 -



ALSO PRESENT

- 1)  $\text{Na}^+/\text{H}^+$  antiporters
- 2) phospholipase C-8
- 3) tensin
- 4) PI - 3 - Kinase
- 5) PI - 4, 5 - Kinase

FIG. 1

- 2 / 6 -

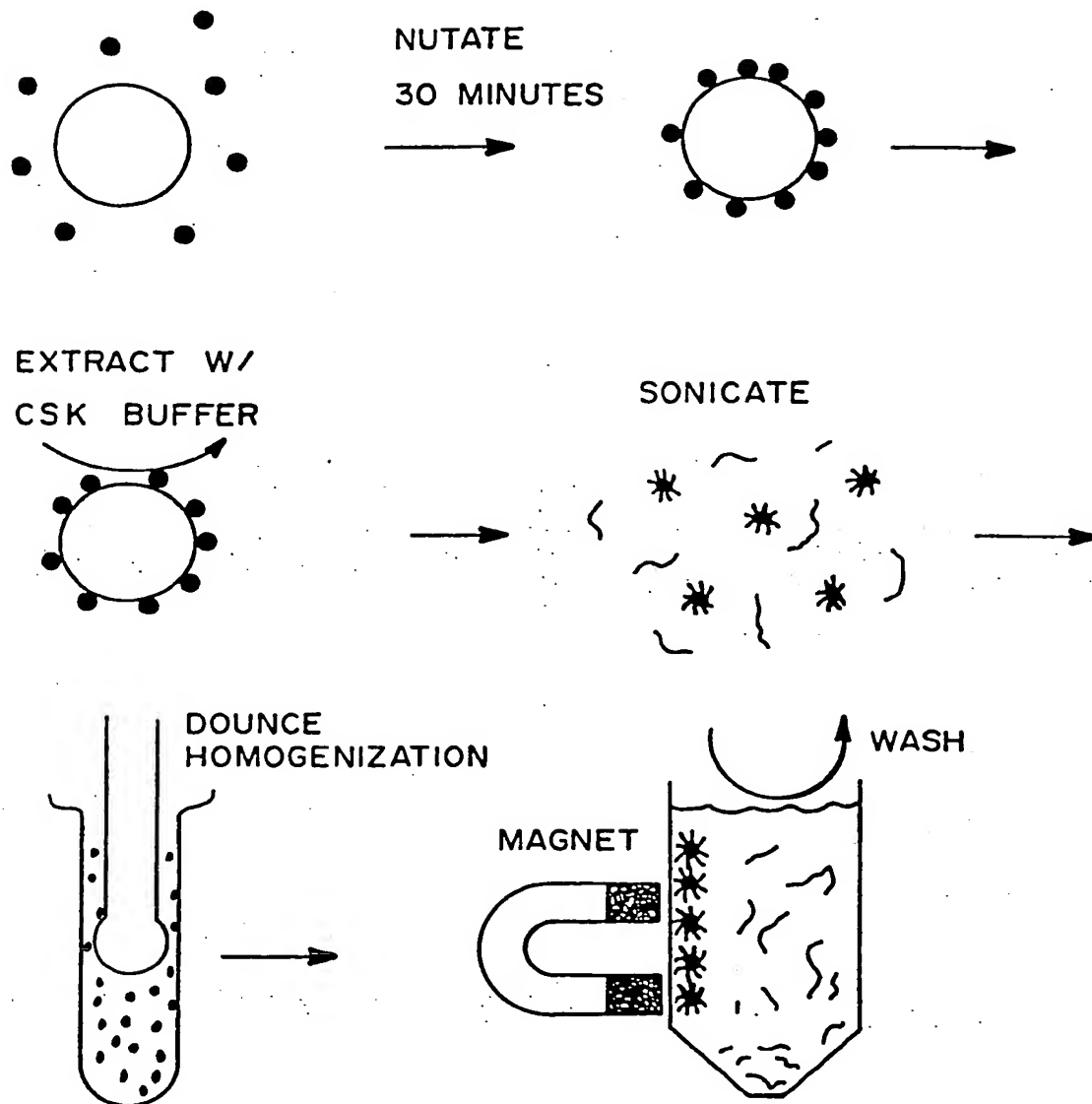


FIG. 2

- 3 / 6 -

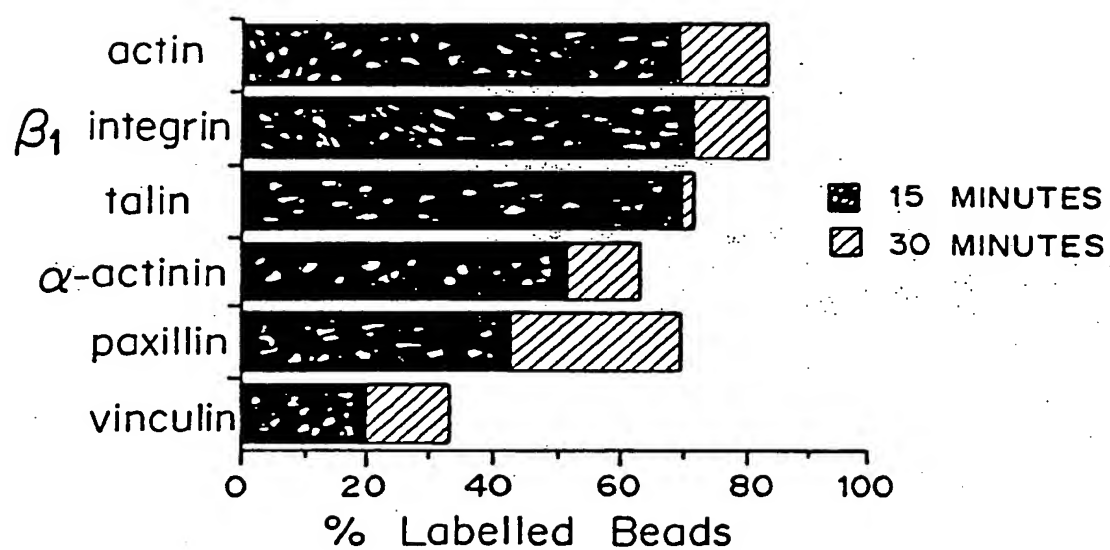
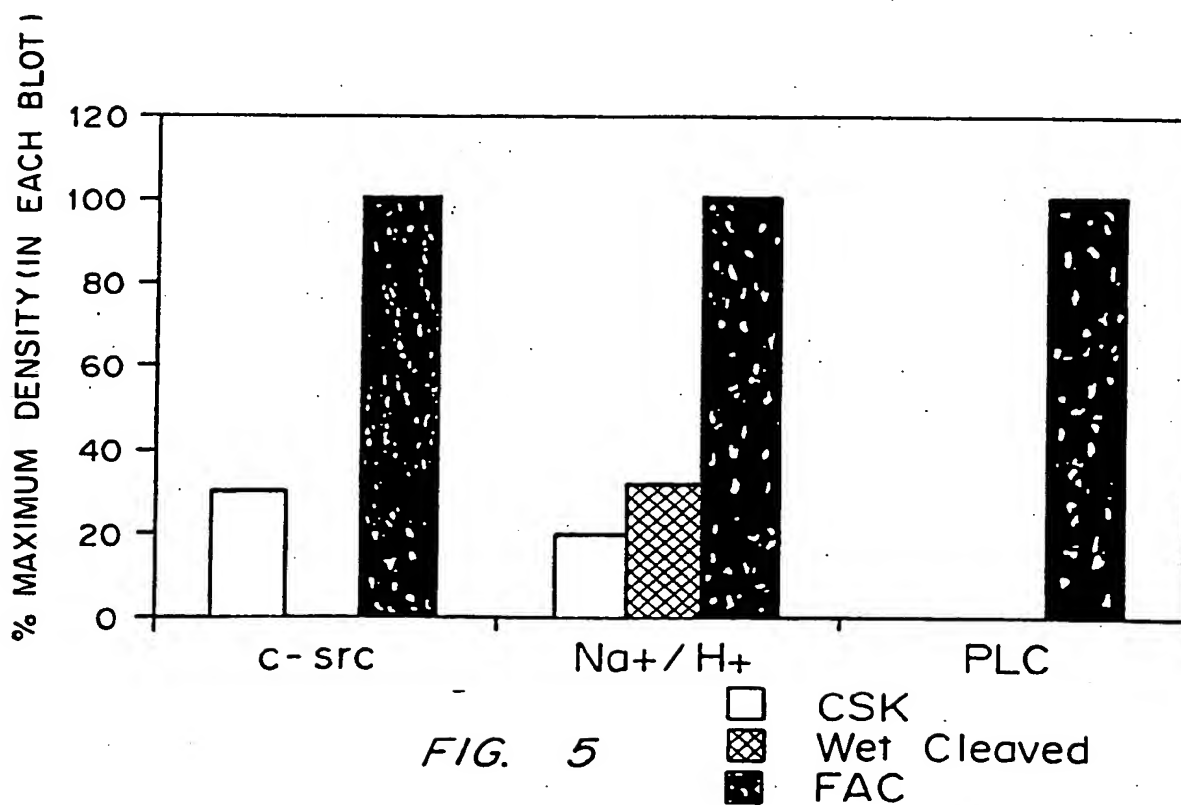
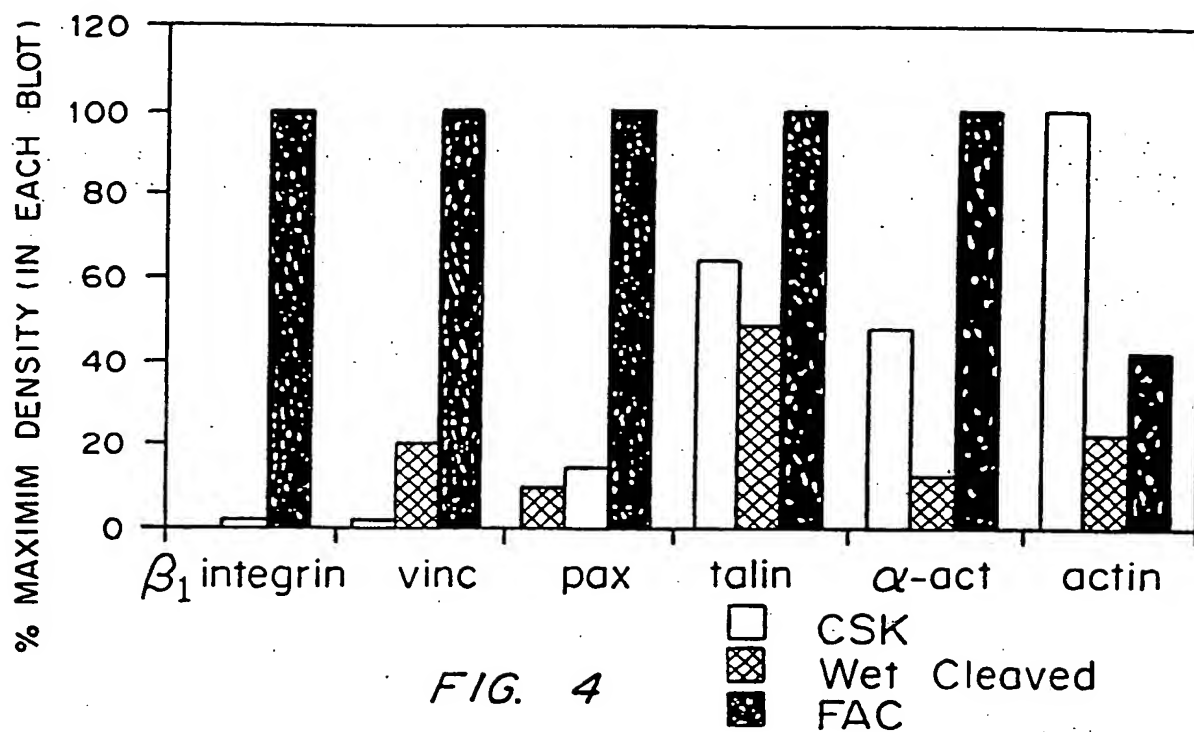


FIG. 3

- 4 / 6 -



- 5 / 6 -

FIG. 6A

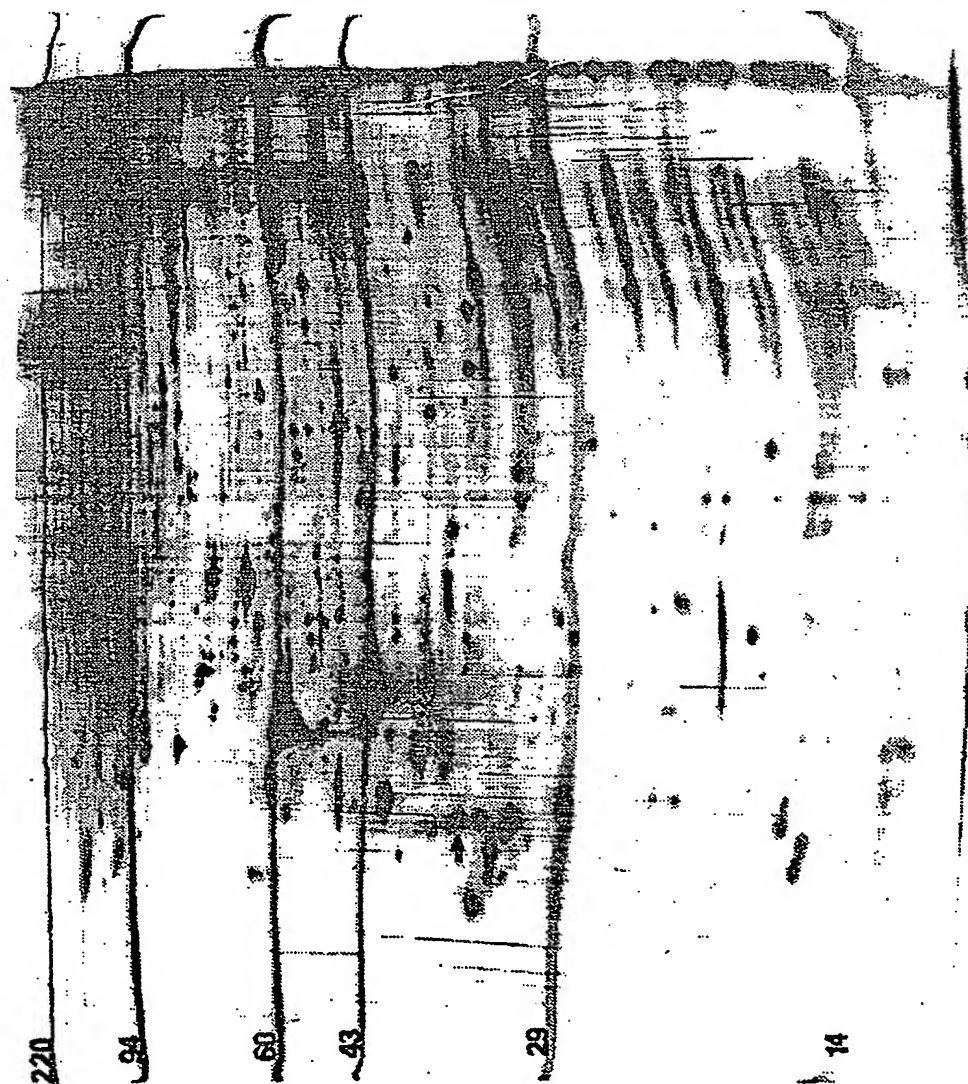
PLO-2, MICRODOMAIN



- 6 / 6 -

FIG. 6B

ING-1, WHOLE CYTOSKELETON



## INTERNATIONAL SEARCH REPORT

Int. l. Application No

PCT/US 94/04481

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 5 C07K15/00 C07K1/14 C07K17/14 C12Q1/68 //C12N15/12

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 5 C12N C12P C07K C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS. vol. 193, no. 2, 15 June 1993, DULUTH, MINNESOTA US pages 571 - 578 PLOPPER G; INGBER DE; 'Rapid induction and isolation of focal adhesion complexes.' see the whole document	1-28
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 87, no. 9, May 1990, WASHINGTON US pages 3579 - 3583 INGBER DE; 'Fibronectin controls capillary endothelial cell growth by modulating cell shape.'	14
A	see the whole document	1-13, 15-28

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

## \* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

22 July 1994

Date of mailing of the international search report

- 1. 08. 94

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,  
Fax (+ 31-70) 340-3016

Authorized officer

Nauche, S



## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 94/04481

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 88, no. 1 , 1 September 1991 , WASHINGTON US pages 7849 - 7853 SCHWARTZ MA;LECHENE C;INGBER DE; 'Insoluble fibronectin activates the Na/H antiporter by clustering and immobilizing integrin alpha 5 beta 1, independent of cell shape.'	14
A	see the whole document	1-13, 15-28
A	--- US,A,4 615 984 (STOCKER, R.L.) 7 October 1986 See the Example, claims 1-9 ---	1-28
A	--- CURRENT OPINION IN CELL BIOLOGY vol. 3, no. 5 , October 1991 pages 841 - 848 INGBER, D. 'Integrins as mechanochemical transducers' cited in the application see the whole document -----	1-28

## INTERNATIONAL SEARCH REPORT

### Information on patent family members

Inte. J. Appl. Application No

PCT/US 94/04481

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A-4615984	07-10-86	NONE	

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☐ **FADED TEXT OR DRAWING**
- ☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☒ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**